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14. ABSTRACT

The original hypothesis was that blocking DR5 with vaccine-induced antagonist antibodies (Ab) will protect T cells from TRAIL-induced apoptosis and enhance their anti-tumor activity. The three specific aims were to (1) Construct and test DR5 vaccines to induce anti-DR5 Ab, (2) Test the antagonist activity of vaccine-induced anti-DR5 Ab, and (3) Amplify anti-tumor immunity by DR5 vaccination. We found that immune sera to human DR5 showed significant agonist, rather than antagonist activity to induce profound tumor cell apoptosis. Histone deacetylase induced the expression of TRAIL in tumor cells via SP-1 and may be used to complement DR5 vaccination. Furthermore, activated T cells were resistant to DR5 mediated apoptosis. Based on these interesting results, we will proceed with the revised hypothesis that agonist DR5 Ab induced by DNA vaccination will trigger tumor cell apoptosis without compromising T cell activity. The specific aims are to (1)Construct and test DR5 vaccines to induce anti-DR5 Ab, (2) Test the agonist activity of vaccine-induced anti-DR5 Ab, and (3) Amplify anti-tumor activity of DR5 vaccination with novel chemotherapeutics.

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INTRODUCTION

The original hypothesis was that blocking TRAIL death receptor DR5 with vaccine-induced antagonist antibodies (Ab) will protect T cells from TRAIL-induced apoptosis and enhance their anti-tumor activity. The three specific aims were

(1) Construct and test DR5 vaccines to induce anti-DR5 Ab,
(2) Test the antagonist activity of vaccine-induced anti-DR5 Ab,
(3) Amplify anti-tumor immunity by DR5 vaccination.

We found that immune sera to human DR5 showed significant agonist, rather than antagonist activity to induce profound tumor cell apoptosis. Furthermore, activated T cells were resistant to DR5 mediated apoptosis. Based on these interesting results, we will proceed with the revised hypothesis that agonist DR5 Ab induced by DNA vaccination will trigger tumor cell apoptosis without compromising T cell activity. The specific aims are to

(1) Construct and test DR5 vaccines to induce anti-DR5 Ab,
(2) Test the agonist activity of vaccine-induced anti-DR5 Ab,
(3) Amplify anti-tumor activity of DR5 vaccination with novel chemotherapeutics.

BODY

Specific Aims 1-2

(1) Construct and test DR5 vaccines to induce anti-DR5 Ab
(2) Test the agonist activity of vaccine-induced anti-DR5 Ab

Part of the results have been reported in
Back, J., Wu, G., Piechocki, MP., Jones, RF., Yagita, H. and Wei WZ., Inhibition of tumor growth by DNA vaccines encoding TRAIL receptor DR5. Proc. AACR, 2008

Mouse DR5 was chosen as the vaccine target because it was the only agonist TRAIL receptor in mice and it was expressed on solid tumors as well as lymphocytes. Humans have, however, two agonist receptors DR4 and DR5. Using modified TRAILs which bind selectively to human DR4 or DR5, signaling through DR5 shows greater apoptotic effect on solid tumors (1). In B cell malignancies, apoptosis is initiated more effectively via DR4 (2) and may indicate a preferential usage of DR4 in cells of hematopoietic origin. To ensure the applicability of our study in patients, human DR5 DNA vaccine was generated and tested.

Agonist activity of anti-human DR5 (hDR5) Ab induced by DNA vaccination

BALB/c mice were electro-vaccinated, 4 times, with naked DNA phDR5 encoding wild type human DR5. Binding of immune sera to hDR5 was verified with MDA-MB231 breast carcinoma cell line which over-expressed DR5 (Fig. 1A). Recognition of hDR5 by the immune sera was further verified by their binding to cells transiently transfected with phDR5 (not shown). To test the functionality of vaccine induced hDR5 Ab, human ovarian cancer line SKOV-3 cells were incubated with control or immune sera (1:8), followed by goat anti-mouse IgG-Fc for cross-linking. More than 70% of treated cells were recognized by Annexin V-PE

(right top and right bottom quadrants), showing apoptosis-associated membrane alteration. Staining by 7-AAD indicated additional nuclear damage of the apoptotic cells (right top quadrants). Normal mouse sera were the negative control. Recombinant human TRAIL (rhTRAIL) or agonist mAb to human DR5 was the positive control. Induction of cell death by hDR5 immune sera was verified in monolayer cultures: cells treated with immune sera, rhTRAIL or hDR5 mAb detached from the monolayer culture (Fig.1C). These results showed that Ab induced by phDR5 vaccination bound to hDR5 on human cancer cells to trigger cell death. Additional hDR5 constructs are being generated and tested to produce a vaccine that can overcome immune tolerance in humans.

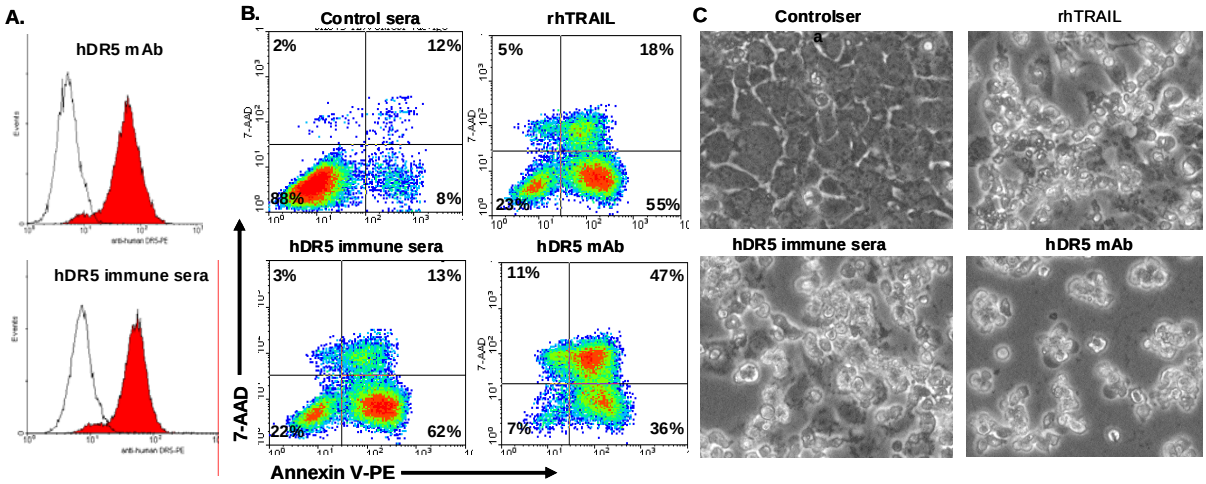

Figure 1. Induction of agonist Ab by hDR5 DNA electrovaccination. BALB/c mice received electrovaccination with pGM-CSF and phDR5 which encoded wild type human DR5. (A) Binding of immune sera to human DR5. MDA-MB231 breast cancer cells were stained with hDR5 mAb or isotype control. Specific binding by hDR5 mAb verified their expression of DR5 (top panel). Incubation with hDR5 immune sera (1:20) or pre-immune sera (lower panel) showed binding of SKOV-3 cells by the immune sera. (B) Induction of apoptosis by hDR5 immune sera. Ovarian cancer SKOV-3 cells were incubated with control or immune sera (1:8) for 2 hrs. Sera was removed and cells washed once before treatment with 2.5 μg/mL cycloheximide and 5 μg/mL goat anti-mouse Fc-specific IgG-FITC for cross-linking. After overnight incubation, cell apoptosis was measured by staining with Annexin V-PE and 7-AAD. Contorl groups were treated with recombinant human TRAIL (rhTRAIL) or agonist hDR5 mAb and they showed comparable apoptosis. Normal mouse sera was the negative control. (C) Induction of cell death in monolayer culture. SKOV-3 cells in monolayer culture were treated with hDR5 immune sera, cycloheximade and secondary Ab as in (B). Cells treated with immune sera, but not control sera, were detached from the monolayer culture. Positive control wells that received rhTRAIL and agonist mAb also showed cell detachment.

Activated T cells are resistant to DR5 mediated apoptosis

We showed in the original application that electro-vaccination of Treg depleted BALB/c mice with pVAX/mDR5-ectm-Td1 encoding mouse DR5 extracellular and transmembrane domains fused to Tetanus toxin fragment C domain 1(Td1) resulted in mDR5 Ab that bound to 3T3 cells transiently transfected with pVAX/DR5-ectm-eGFP to express both DR5-ectm and eGFP in the same cell. However, mouse mammary tumor cells D2F2 incubated with mDR5 immune sera, followed by cross-linking with goat α-mouse Ig, did not undergo apoptosis. But, a modest inhibition of tumor growth was observed in mDR5 vaccinated mice. These results may indicate modest agonist activity that triggers tumor cell apoptosis, or antagonist activity that prevents immune cell apoptosis.

Since DR5 is the only agonist TRAIL death receptor in mice, it may mediate apoptosis of both tumor cells and activated immune cells. We compared the induction of apoptosis in mouse mammary tumor cells D2F2 and in T cells that were activated by anti-CD3 and IL-2. Resting T cells expressed little DR5 which was elevated after cells were treated with anti-CD3 and IL-2 to reach a comparable level as that of D2F2 tumor cells (Figure 2A, insert). Treatment with anti-DR5 mAb MD5-1 and TRAIL induced significant apoptosis in D2F2 cells when measured by

Annexin V binding or MTT assay (Figure 2A-B). Activated T cells, on the other hand, did not show increased apoptosis when incubated at 2X concentration of mAb and TRAIL. Even when XIAP inhibitor Embelin was added to dampen possible effect from the apoptosis inhibitor, little apoptosis was observed in activated T cells (not shown). These results would argue that the majority of T cells are relatively resistant to DR5 mediated apoptosis and may not be affected by anti-DR5 Ab.

We will continue to analyze the agonist activity of DR5 immune sera and whether induction of tumor cell apoptosis results in amplified priming to tumor-associated antigens. mDR5 will be used to develop a candidate vaccine formulation that can overcome immune tolerance to induce high level DR5 binding Ab. This vaccine formulation will be used to guide the generation of human DR5 vaccine.

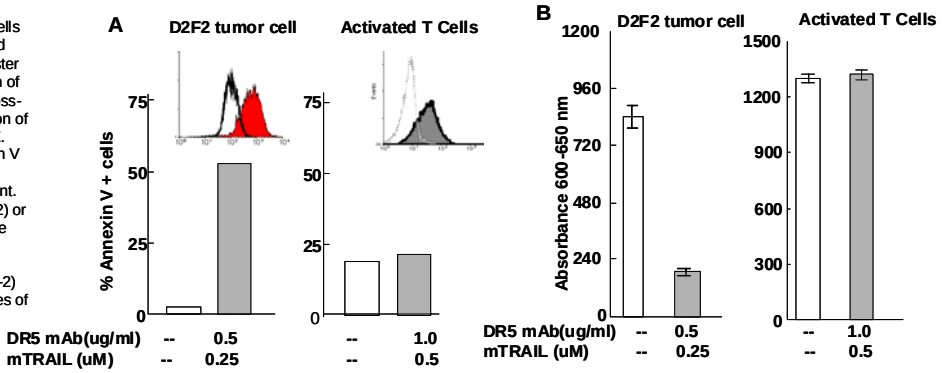

Figure 2 Selective induction of apoptosis in tumor cells by anti-DR5 mAb and TRAIL. D2F2 cells were treated with 0.5 μg/ml anti-DR5 mAb, MD5-1 or normal hamster isotype control for 30 min at 37°C prior to the addition of 0.25μg/mL recombinant soluble mouse TRAIL for cross-linking and the cells were incubated for 20h. Induction of apoptosis was evaluated by binding of Annexin V-PE. Bars represent the percent of cells binding to Annexin V as detected by flow cytometry. In parallel, cells were tested for proliferative activity at 24-48h after treatment. Absorbance data detect the metabolite of MTT (D2F2) or MTS (T cells) produced by proliferating cells. Data are mean+SEM of quadruplicate wells. Activated T cell cultures (Day 7-10 following initial activation on immobilized anti-CD3 and expansion in 1200 U/ml IL-2) were treated in an identical fashion using higher doses of MD5-1 (1.0 μg/mL) and TRAIL (0.5 μg/mL).

Specific Aim 3 Amplify anti-tumor activity of DR5 vaccination with novel chemotherapeutics.

Part of the results have been reported in
Xu, J., Zhou, J.Y., Wei,Z.W., Philipsen, S., and Wu G.S. Sp1-mediated TRAIL induction in chemo-sensitization. Cancer Res 68(16):6718-26, 2008.

Histone deacetylase (HDAC) inhibitors can complement DR5 targeted therapy

Because hDR5 immune sera induce significant tumor cell apoptosis, it may be advantageous to combine hDR5 vaccination with conventional or novel chemotherapeutics, particularly those agents that induce the expression of TRAIL and TRAIL receptors by tumor cells. Histone deacetylase (HDAC) inhibitors are a new class of anticancer agents that activate the transcription of target genes via histone acetylation. It has been reported in the literature that HDAC inhibitors induce DR5 and sensitize cancer cells to TRAIL-mediated cell death (3), suggesting their complementary activity with DR5 vaccination. We examined and demonstrated the induction of TRAIL in human breast cancer cells by histone deacetylase (HDAC) inhibitor MS275 or SAHA. Furthermore, induction of TRAIL by HDAC inhibitor MS275 was enhanced by conventional chemotherapeutic agent, Adriamycin. To examine the mechanism of TRAIL induction, different reporter constructs in conjunction with transcription activity assays and chromatin immunoprecipitation assays were used to show that the transcription factor Sp1 mediated the induction of TRAIL by MS275 alone or in combination with adriamycin.

Treatment of breast cancer with MS275 and Adriamycin increased apoptosis via death receptor (extrinsic) as well as mitochondrial (intrinsic) apoptotic pathways. Down-regulation of TRAIL by small interfering RNA silencing decreased MS275-mediated Adriamycin-induced caspase activation and apoptosis, further demonstrating the apoptotic effect of TRAIL induced by drug treatment. Importantly, breast cancer cells T47D in which Sp1 was knocked down or Sp1-knockout mouse embryonic stem cells were resistant to the combined drug treatment.

In mouse mammary tumor cells that showed reduced sensitivity to DR5 mediated apoptosis, treatment of HDAC inhibitors amplified the anti-tumor effect. Taken together, our results indicate that Sp1 mediates the induction of TRAIL in cells treated with MS275 and Adriamycin and these drugs may be used to complement hDR5 vaccination.

KEY RESEARCH ACCOMPLISHMENTS

1. Generation of agonist Ab to human DR5 by DNA vaccination
2. Establish HDAC inhibitor as appropriate complementary treatment with DR5 vaccination.

REPORTABLE OUTCOMES

Back, J., Wu, G., Piechocki, MP., Jones, RF., Yagita, H. and Wei WZ., Inhibition of tumor growth by DNA vaccines encoding TRAIL receptor DR5. Proc. AACR, 2008

Xu, J., Zhou, J.Y., Wei,Z.W., Philipsen, S., and Wu G.S. Sp1-mediated TRAIL induction in chemo-sensitization. CancerRes 68(16):6718-26, 2008.

US patent application in preparation “Cancer vaccines targeting TRAIL death receptors”

CONCLUSIONS

Agonist Ab to human DR5 is induced by electrovaccination with human DR5 DNA to induce tumor cell apoptosis. Activated T cells are relatively resistant to DR5 mediated apoptosis, indicating feasibility of using DR5 vaccination for cancer control. Induction of tumor cell apoptosis by DR5 immune sera may facilitate in situ priming to tumor associated antigens and will be pursued. HDAC inhibitor induces DR5 and TRAIL expression of treated tumor cells, the latter via the transcription factor Sp-1. Combining DR5 vaccination with HDAC inhibitor or other TRAIL/DR5 inducing chemotherapeutic agents may be a strong treatment modality for cancer control.

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Appendix 1

2008 AACR

Inhibition of tumor growth by DNA vaccines encoding TRAIL receptor DR5

J.B. Back[1], G.S. Wu[1], R.F. Jones[1], M.P. Piechocki[1], H. Yagita[2], and W.Z. Wei[1]
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The TNF-related apoptosis-inducing ligand (TRAIL/Apo2L), a member of the TNF family, induces apoptosis in many tumor cell lines through cognate death receptors (DRs). These DRs possess intracellular death domains which activate both mitochondria-dependent and –independent apoptosis pathways through FADD-caspase-8 and subsequent activation of the caspase cascade. TRAIL or agonist Ab to DRs preferentially induces apoptotic tumor cell death in preclinical models; however, TRAIL resistant cancers are often encountered. Results from Phase I/II clinical trials with mAb to human DR4/5 show limited toxicity and stable disease in some cases, suggesting that DRs may be viable vaccine targets. To test this hypothesis, BALB/c mice were depleted of regulatory T cells and immunized 3-4 times with DNA encoding the extracellular and transmembrane domains of mouse DR5 (mDR5) fused at the C-terminus with domain 1 of tetanus toxin fragment C (pmDR5ectm-Td.1). Resulting anti-mDR5 Abs were detected by flow cytometry via specific binding to mDR5/GFP-expressing cells. Immunization with heterologous human or rat DR5 DNA did not induce Ab that recognized mouse DR5. A xenogeneic cell vaccine overexpressing mDR5ectm-Td.1 has also been generated which boosts anti-mDR5 Ab response following DNA vaccination. Approximately 30% of pmDR5ectm-Td.1 immunized BALB/c mice were protected from a subsequent challenge with D2F2 or TUBO mammary tumor cells which endogenously express mDR5 suggesting DNA vaccination may protect mice from tumor growth either alone or in combination with chemotherapy. Since TRAIL is also implicated in mediating immune surveillance and thymocyte apoptosis and because DR5 expression is upregulated on activated T cells, the mechanism of tumor rejection by mDR5 vaccination may be direct killing of tumor cells by DR5 Ab and/or indirectly via T cell modulation. A thorough investigation of the mechanism(s) is warranted. (CA76340 and DOD W81XWH-07-1-0521)

Sp1-Mediated TRAIL Induction in Chemosensitization

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Abstract

The regulation of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in cancer chemotherapy is not fully understood. Here, we show that the histone deacetylase (HDAC) inhibitors induce TRAIL in human breast cancer cells. Induction of TRAIL by the HDAC inhibitor MS275 can be enhanced by Adriamycin. Using different reporter constructs in conjunction with transcription activity assays and chromatin immunoprecipitation assays, we provide evidence that the transcription factor Sp1 is responsible for TRAIL induction by MS275 alone or in combination with Adriamycin. Further, we show that the combined treatment of breast cancer cells with MS275 and Adriamycin significantly increases apoptotic cell death via the activation of both death receptor and mitochondrial apoptotic pathways. Down-regulation of TRAIL by small interfering RNA silencing decreased MS275-mediated Adriamycin-induced caspase activation and apoptosis, thus conferring Adriamycin resistance. More importantly, breast cancer T47D cells in which Sp1 was knocked down or Sp1-knockout mouse embryonic stem cells were resistant to the combined treatments. Taken together, our results indicate that induction of TRAIL by the combined treatments with MS275 and Adriamycin is mediated by Sp1 and suggest that transcription factor Sp1 is an important target for the development of novel anticancer agents. [Cancer Res 2008;68(16):6718–26]

Introduction

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) is a member of the TNF family (1, 2). TRAIL selectively induces apoptosis of transformed or tumor cells but not normal cells, making it a promising new agent for cancer therapy (1, 2). There are four membrane-bound receptors for TRAIL: DR4 (3), DR5 (4–8), TRAIL-R3 (6, 9–11), and TRAIL-R4 (12, 13). DR4 and DR5 both contain a conserved death domain motif and are proapoptotic receptors (14), whereas TRAIL-R3 lacks an intracellular domain and TRAIL-R4 has a truncated death domain. Thus, both TRAIL-R3 and TRAIL-R4 act as decoy receptors to antagonize TRAIL-induced apoptosis by competing for ligand binding (14, 15). Binding of TRAIL to DR4 or DR5 triggers formation of the death-inducing signaling complex by recruiting Fas-associated death domain and caspase-8 or caspase-10, resulting in the activation of caspase-8 or caspase-10, which in turn activates caspase-3, caspase-6, and caspase-7 to cleave death substrates and cause cell death. Activated caspase-8 can cleave the proapoptotic Bcl2 family member Bid to generate truncated Bid. Truncated Bid translocates to the mitochondria to cause cytochrome *c* release, which amplifies the apoptotic signal from the TRAIL pathway.

It has been shown that DR5 could be transcriptionally induced by some anticancer drugs (4, 16), thus sensitizing cancer cells to TRAIL (16, 17). However, the regulation of TRAIL ligand expression is much less understood. The TRAIL promoter contains a number of transcription regulatory elements including IFN-stimulated response element, nuclear factor κB, and Sp1 (18, 19). It has been shown that IFNs directly induce TRAIL in both human leukemia Jurkat and colon cancer HT29 cells (18, 19). IFN-γ also acts as a mediator to induce TRAIL in response to retinoid treatment (20). The induction of TRAIL via an IFN-stimulated response element results in apoptosis (20, 21). In addition, the TAX oncoprotein encoded by human T-cell leukemia virus induces TRAIL through the nuclear factor-κB–dependent pathway (22). TRAIL is also induced by T-cell receptor mimetics in human T cells, and such induction involves a c-Rel binding site in the proximal TRAIL promoter (23). However, the regulation of TRAIL by cancer chemotherapy is not fully understood.

The histone deacetylase (HDAC) inhibitors are novel anticancer agents that can activate transcription of target genes via histone acetylation (24). By activation of gene expression, HDAC inhibitors may induce cell differentiation, growth arrest, and apoptosis. The ability of HDAC inhibitors to induce apoptosis is attributed to the activation of both extrinsic and intrinsic apoptotic pathways (25). Several HDAC inhibitors, including valproic acid, suberoylanilide hydroxamic acid (SAHA), and the benzamide derivative MS275, exhibit antitumor activity with little toxicity to normal cells both *in vitro* and *in vivo* (24). Recent studies have shown that HDAC inhibitors induce leukemia-selective apoptosis through the TRAIL apoptotic pathway (26, 27) and sensitize leukemia cells to anticancer agents (28). In addition, it has been shown that HDAC inhibitors induce DR5 and subsequently sensitize cancer cells to TRAIL-mediated cell death (29). How activation of TRAIL pathway contributes to HDAC inhibitor–induced apoptosis in solid tumors is of critical importance and needs to be determined.

In this article, we show that the HDAC inhibitor MS275 induces TRAIL via an Sp1-dependent pathway. MS275-mediated TRAIL induction was enhanced by Adriamycin at both the RNA and protein levels. The induction of TRAIL by MS275 enhanced Adriamycin-induced apoptosis in human breast cancer cells. Down-regulation of TRAIL by small interfering RNA (siRNA) decreased Adriamycin-mediated cell death induced by MS275. Importantly, T47D cells in which Sp1 was knocked down or Sp1 null embryonic stem cells were more resistant to Adriamycin,

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MS275, or combination treatment as compared with their counterparts with intact Sp1. Thus, our data indicate that Sp1-mediated TRAIL induction plays a critical role in chemosensitivity and suggest that Sp1 is a therapeutic target for the development of novel anticancer therapeutics.

Materials and Methods

Reagents. MS275 was purchased from Alexis Biochemicals. SAHA was purchased from Cayman. Adriamycin was obtained from the Oncology Outpatient Pharmacy at the Karmanos Cancer Institute. Monoclonal antihuman TRAIL, DR4, and polyclonal DR5 antibodies were purchased from Imgenex. Rabbit anti–caspase-9, anti–caspase-8, anti–caspase-3, and anti–poly(ADP-ribose) polymerase (PARP) polyclonal antibodies were purchased from Cell Signaling Technology. Monoclonal p21 antibody was purchased from Calbiochem. Anti-actin antibody was purchased from Sigma. Sp1 antibody was purchased from Upstate Biotechnology.

Cell lines, culture conditions, and treatment. The human breast cancer MCF7 cells were obtained from Karmanos Cancer Institute and maintained in DMEM/F-12. The human breast cancer MDA231 and T47D cells were obtained from American Type Culture Collection and maintained in DMEM. Cells were supplemented with either 10% fetal bovine serum (FBS) for MDA231 and T47D or 5% FBS for MCF7 and antibiotics at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. Sp1-knockout mouse embryonic stem cells ($Sp1^{-/-}$) and their normal control embryonic stem cells ($Sp1^{+/+}$) were previously described (30) and maintained in Glasgow MEM (Sigma) supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1× nonessential amino acids, 10% FBS, β-mercaptoethanol, and leukemia inhibitory factor (Chemicon). Embryonic stem cells were grown in bovine gelatin–coated dishes.

Isolation of RNA and Northern blot analysis. The procedures for preparation of total RNA and Northern blot analysis were previously described (31).

siRNA transfection for knockdown of TRAIL and Sp1. On-TARGETplus SMARTpool siRNAs for TRAIL, Sp1, and corresponding control siRNA were purchased from Dharmacon Research. The transfection was done as suggested by Dharmacon with slight modifications, as described previously (32). Briefly, T47D cells were plated at 6×10^5 per well in six-well plates. The next day, cells were transfected with TRAIL, Sp1, or nontarget control oligonucleotides using Oligofectamine (Invitrogen). After 3 d, transfected cells were left untreated or treated with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 48 h and then harvested for assessing the expression of TRAIL and Sp1 and the activation of the caspase cascade by Western blot analysis. To determine chemosensitivity, cells with or without transfections were placed at 8,000 per well in 96-well plates and then treated with MS275, Adriamycin, or their combination for 48 h, and cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

MTT assays. MTT assays were described previously (33).

Western blot analysis. The procedures for preparation of whole-cell protein lysates and Western blot analysis were described previously (33).

Assay of caspase-3 activity. The enzymatic activity of caspase-3 was assayed using the caspase-3 colorimetric assay kit (R&D Systems) according to the manufacturer's protocol. Cells were left untreated or treated with MS275, Adriamycin, or their combination for 48 h and then lysed in lysis buffer for 10 min on ice. The lysed cells were centrifuged at 14,000 rpm for 5 min, and 150-μg protein was incubated with 50 μL of reaction buffer and 5 μL of caspase-3 substrate at 37°C for 2 h, and the absorbance was measured at a wavelength of 405 nm on a plate reader.

Construction of reporter vectors. TRAIL reporter constructs pGL3-TRAIL2, pGL3-TRAIL5, and pGL3-TRAIL6 were previously described (33). TRAIL5mu in which the second Sp1 binding site was mutated was amplified from pGL3-TRAIL2 using GC-Rich PCR system (Roche Molecular Biochemicals) and the primers 5′-CCGCTCGAGAGGAAATTTTCTTTACAGTT-3′ and 5′-CCCAAGCTTGATCCTGTCAGAGTCTGACTGCTG-3′. The PCR conditions were as follows: 95°C/3 min; 30 cycles at 95°C/30 s, 45°C/30 s, and 68°C/2 min; followed by 68°C/7 min for the final extension. The amplified fragment was isolated from 1% agarose gel, digested with *Xho*I and *Hin*dIII, and subcloned into pGL3-Basic (Promega). The insert was verified by DNA sequencing.

Luciferase reporter assays. Transfections for luciferase assays were carried out as described previously (33). Briefly, T47D cells were plated at 8×10^5 per well in six-well plates. The next day, the cells were transfected with 5 μg of reporter constructs and 5 ng of pRLSV40 (Promega) using Lipofectamine 2000 reagent (Invitrogen). After 24 h, transfected cells were treated with or without 5 μmol/L MS275, 0.1 μg/mL Adriamycin, or their combination for 24 h. Firefly luciferase activities were assayed using the dual-luciferase reporter assay system (Promega) in a Turner TD20/20 luminometer and normalized to Renilla luciferase activity.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was done with the ChIP Assay Kit (Upstate Biotechnology) as described previously (33). The PCR primers used in chromatin immunoprecipitation were 5′-AATGGGCTTGAGGTGAGTGCAGAT-3′ and 5′-ATGAGTTGTTTT-TCTGGGTTCTGT-3′.

ELISA for Sp1 transcription activity. T47D cells were left untreated or treated with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 24 h and nuclear protein was extracted with a Nuclear Extraction kit (Panomics). Nuclear protein was then quantified using the Bio-Rad Protein Assay kit (Bio-Rad). A total of 15 μg of nuclear protein from each treatment were analyzed for Sp1 activity using the Transcription Factor ELISA kit (Panomics). Sp1 antibody was used as primary antibody and antirabbit IgG horseradish peroxidase was used as secondary antibody. The absorbance was measured at a wavelength of 450 nm on a spectrophotometer.

Statistical analysis. Statistical analysis was done using Student's t test. The data were presented as the mean ± SD, and $P \leq 0.05$ was considered significant.

Results

HDAC inhibitors induce TRAIL expression in human breast cancer cells. The regulation of TRAIL by cancer chemotherapy in solid tumor cell lines is not fully understood. We treated three breast cancer cell lines, MDA231, T47D, and MCF7, with various doses of MS275 for 24 hours, and induction of TRAIL was determined by Western blot analysis. Figure 1 shows that TRAIL protein was induced in all three lines at different doses of MS275 and that such treatments seem to have no effect on the levels of DR4 and DR5 proteins except for an increase in DR5 protein in MCF7 cells treated with 5 μmol/L MS275. Consistent with previous studies (26, 27), p21 was induced by MS275, which served as a positive control. TRAIL mRNA was also increased by MS275 treatment (Fig. 1*B*). Further, we found that SAHA, another HDAC inhibitor, can also induce TRAIL expression (Fig. 1*A*). These data suggest that TRAIL induction by HDAC inhibitors is a common event in breast cancer cells.

MS275 sensitizes human breast cancer cells to Adriamycin. We previously showed that induction of TRAIL by TNFα and 5-aza-2′-deoxycytidine plays a critical role in sensitizing breast cancer cells to chemotherapy (33, 34). To determine the role of TRAIL induction in breast cancer cell death induced by Adriamycin, we first tested the effect of MS275 treatment alone on the growth of breast cancer cells. As shown in Fig. 2*A*, MS275 inhibited the growth of all three cell lines in a dose-dependent manner. We next asked whether the effect of MS275 on growth inhibition could be enhanced by anticancer agents. Figure 2*B* shows that the growth inhibition was ~86% in cells treated with MS275 in combination with Adriamycin, as compared with ~50% and ~33% in cells treated with MS275 and Adriamycin alone, respectively. These data suggest that MS275 could sensitize T47D cells to Adriamycin.

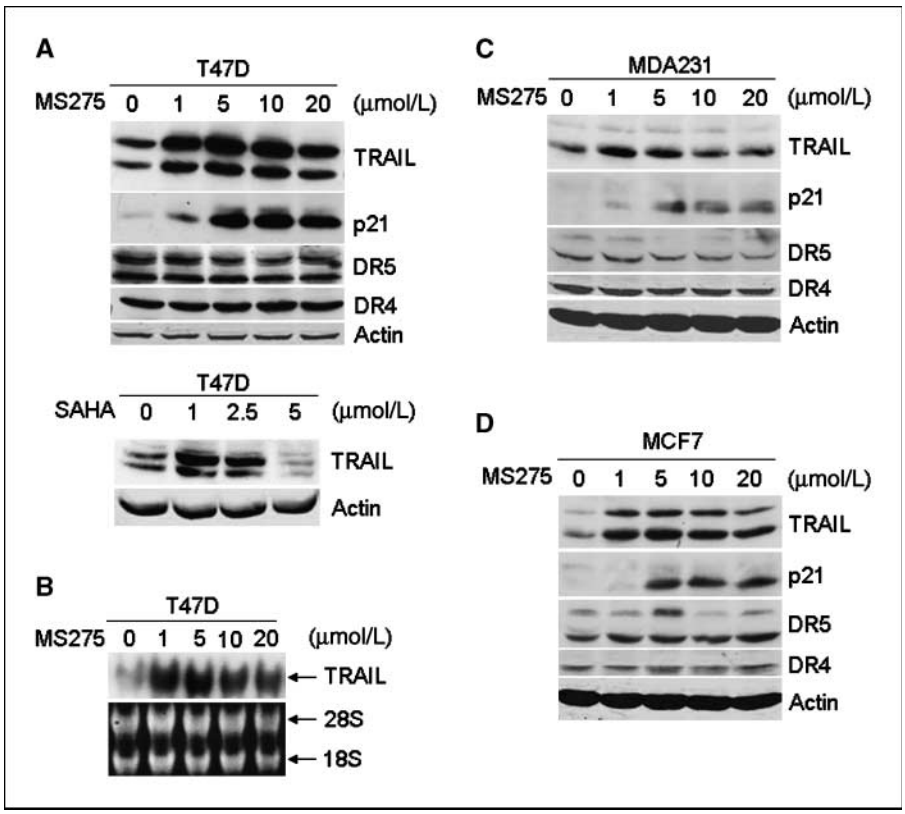

Figure 1. Induction of TRAIL by HDAC inhibitors. *A, C,* and *D,* effects of MS275 or SAHA on TRAIL, DR4, DR5, and p21 proteins. T47D (*A*), MDA231 (*C*), and MCF7 (*D*) cells were left untreated or treated with various doses of MS275 or SAHA for 24 h, and total protein was then extracted for assaying the expression of TRAIL, DR4, DR5, and p21 by Western blot analysis. β-Actin was used as a loading control. *B,* induction of TRAIL mRNA by MS275 in T47D cells. Cells were treated as in *A*, and total RNA was extracted to examine for TRAIL mRNA expression by Northern blot analysis. rRNA was used as a loading control.

To determine the underlying mechanisms by which MS275 sensitizes T47D cells to Adriamycin, we tested the activation of the apoptotic pathways because HDAC inhibitors can kill cancer cells by apoptosis (25). Figure 2*C* shows that Adriamycin or MS275 treatment alone causes modest cleavage of caspase-9 and PARP. In contrast, the combination of 5 μmol/L MS275 with 0.1 μg/mL Adriamycin significantly enhanced cleavage of caspase-9, caspase-3, and PARP (Fig. 2*C*). Importantly, the combined treatments resulted in a significant increase in cleavage of caspase-8, which was not obvious in cells treated with either agent alone (Fig. 2*C*). In addition, the combined treatment also enhanced caspase-3 activity (Fig. 2*D*) relative to the treatments with either agent alone. Collectively, these data suggest that the enhanced cell killing by the combined treatments is attributable to the augmented induction of apoptosis.

Sp1 is responsible for TRAIL induction by MS275 alone or in combination with Adriamycin. To define the mechanisms of TRAIL regulation by MS275, we transfected T47D cells with either the TRAIL luciferase reporter construct pGL3-TRAIL2 or empty vector pGL3-Basic, followed by treatment with MS275 (5 μmol/L). Cells were harvested after 24 hours, and luciferase activity was assayed using the dual-luciferase reporter assay system. Figure 3*B* shows that transfections of pGL3-TRAIL2 containing a 504-bp fragment upstream of translational start site result in an ~2.5-fold increase in luciferase activity in response to MS275 treatment, as compared with untreated cells. To understand TRAIL regulation by MS275 in detail, we tested the effects of MS275 on luciferase activity using several deletion constructs (Fig. 3*A*). As shown in Fig. 3*B*, pGL3-TRAIL5 containing two Sp1 sites was still activated by MS275 whereas pGL3-TRAIL6 with one Sp1 binding site was inert. This suggests that the second Sp1 is critical for MS275-mediated TRAIL induction. To further confirm this, we mutated the second Sp1 site in pGL3-TRAIL5mu and found that pGL3-TRAIL5mu is no longer responsive to MS275 treatment, suggesting that the second Sp1 binding site is responsible for transactivation of the TRAIL promoter by MS275.

Because MS275 could sensitize T47D cells to Adriamycin (Fig. 2), we asked if the combination of MS275 with Adriamycin has any effects on the TRAIL promoter. To answer this question, T47D cells transfected with TRAIL reporter constructs were treated with either agent alone or in combination and then assayed for the TRAIL promoter activity. As shown in Fig. 3*B*, although Adriamycin alone had no effects on the TRAIL promoter activity, the combination of MS275 with Adriamycin resulted in a more pronounced increase in the activation of the TRAIL promoter as compared with cells treated with MS275 alone. Similar to the results obtained with MS275, loss of the second Sp1 binding site abolished the activation of the TRAIL promoter by the combined treatments (Fig. 3*B*). Importantly, we showed that the combined treatments increase the level of TRAIL protein, as compared with cells treated with either agent alone (Fig. 3*C*). Interestingly, in spite of the fact that Adriamycin alone had no effect on the activation of the TRAIL promoter, Adriamycin could augment MS275-mediated activation of the TRAIL promoter, suggesting that this Adriamycin-mediated effect may be indirect. Collectively, these results indicate that the second Sp1 site is critical for the activation of the TRAIL promoter by MS275 alone or in combination with Adriamycin.

To determine whether Sp1 binds directly to the TRAIL promoter in response to MS275 or the combined treatment, we conducted chromatin immunoprecipitation analysis of the TRAIL promoter with anti-Sp1 antibody. T47D cells were left untreated or treated

with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination and then harvested 24 hours later for the chromatin immunoprecipitation experiments. Figure 4*A* and *B* shows that PCR amplification of the immunoprecipitated chromatin with Sp1 antibody results in single bands of a size expected for the TRAIL promoter. Importantly, there were higher levels of amplified DNA in MS275-treated cells than observed in untreated cells. Moreover, the combined treatment resulted in more robust DNA amplification than that of cells treated with MS275 alone. In addition, although Adriamycin did not activate TRAIL promoter constructs (Fig. 3*B*), we observed a slight increase in DNA amplification in Adriamycin-treated cells as compared with untreated cells. To further investigate the activation of Sp1 transcription, we extracted nuclear proteins from cells treated with MS275, Adriamycin, or their combination and used ELISA kit to assay the Sp1 transcription activity. As shown in Fig. 4*C*, either MS275 or Adriamycin treatment resulted in a modest increase in Sp1 transcription activity as compared with untreated cells. In contrast, the combination of MS275 and Adriamycin resulted in an ~4-fold increase in Sp1 transcription activity. Taken together, these results suggest that the induction of TRAIL by MS275 alone or in combination with Adriamycin is mediated by the transcription factor Sp1.

Induction of TRAIL by MS275 is required for sensitization of T47D cells to Adriamycin-induced apoptosis. We have shown that MS275 induces TRAIL and that the combination of MS275 and Adriamycin enhances cell death relative to either agent alone. We asked whether TRAIL induction by MS275 is required for this

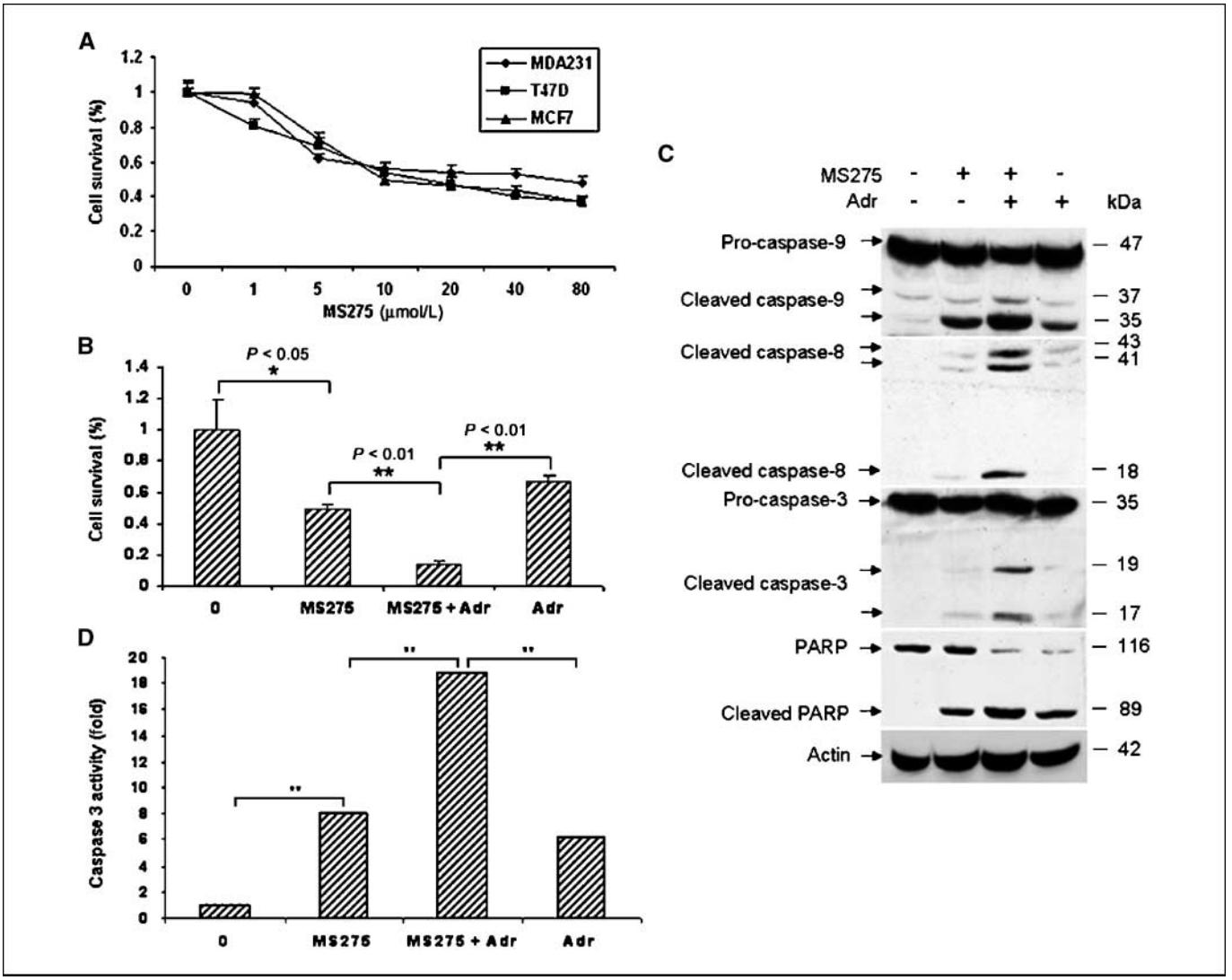

Figure 2. Effect of MS275, Adriamycin, or their combination on cell death. *A,* role of MS275 in growth inhibition. The human breast cancer cell lines T47D, MDA231, and MCF7 were left untreated or treated with various doses of MS275 for 48 h. Cell viability was determined by MTT assays. Representative of three independent experiments. *B,* role of MS275 in T47D cell death induced by Adriamycin. T47D cells were treated with 5 μmol/L MS275, 0.1 μg/mL Adriamycin (*Adr*), or their combination for 48 h. Cell viability was determined by MTT assays. Representative of three independent experiments. **, $P < 0.01$. *C,* activation of the apoptotic pathways by MS275, Adriamycin, or their combination. T47D cells were left untreated or treated with 5 μmol/L MS275, 0.1 μg/mL Adriamycin, or their combination, and total protein was extracted at 48 h. Cleavage of caspase-9, caspase-8, caspase-3, and PARP was determined by Western blot analysis. β-Actin was used as a loading control. *D,* role of the treatments with MS275, Adriamycin, or their combination in caspase-3 activity. T47D cells were treated as in *C* and then lysed in lysis buffer. Cell protein was extracted and used for measuring caspase-3 activity. Representative of three independent experiments. **, $P < 0.01$.

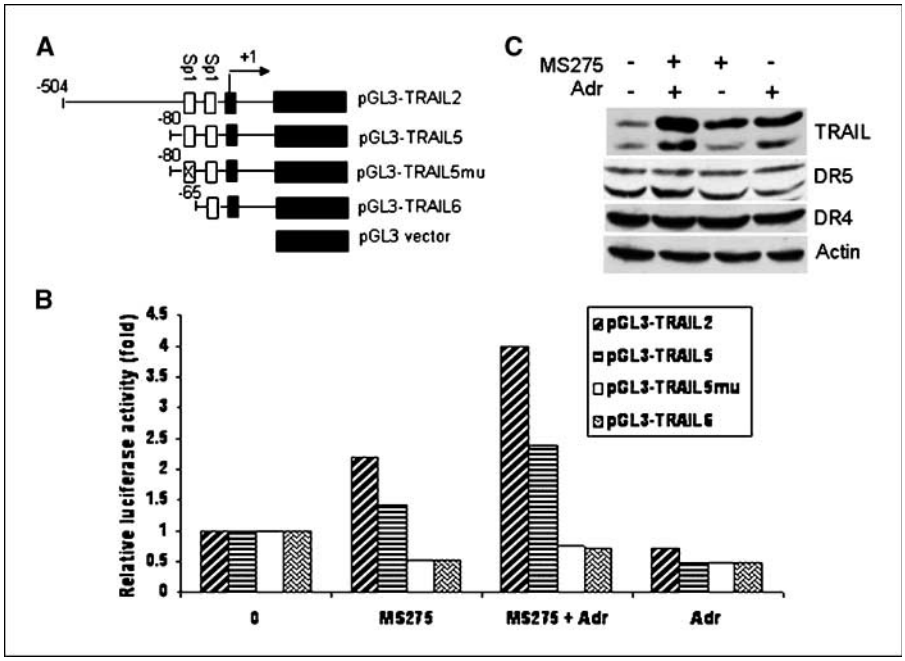

Figure 3. Effect of MS275, Adriamycin, or their combination on the TRAIL promoter activity. *A,* schematic depiction of luciferase reporter constructs. The translation start site is indicated by an arrow and designated at +1 and both Sp1 binding sites were indicated. *B,* activation of the TRAIL promoter by MS275, Adriamycin, or their combination. T47D cells were transfected with pGL3-TRAIL2, pGL3-TRAIL5, pGL3-TRAIL5mu, pGL3-TRAIL6, and pGL3-Basic. pRLSV40 was added in transfections for normalization. The next day, cells were left untreated or treated with 5 μmol/L MS275, 0.1 μg/mL Adriamycin, or their combination. Luciferase activity was determined 24 h later. Data are shown as relative firefly luciferase activities, normalized to Renilla luciferase activities. *C,* induction of TRAIL protein. T47D cells were left untreated or treated with 5 μmol/L MS275, 0.1 μg/mL Adriamycin, or their combination for 24 h. Induction of TRAIL, DR4, and DR5 proteins was assessed by Western blot analysis. Actin was used as a loading control.

enhanced killing effect. To this end, we transfected T47D cells with either control siRNA or siRNA against TRAIL, and then tested the effect of siRNA-mediated TRAIL down-regulation on cell death. As shown in Fig. 5*A*, the basal levels of TRAIL protein in cells transfected with TRAIL siRNA were decreased as compared with cells transfected with control siRNA. Further, the induction of TRAIL by MS275 was also decreased in cells transfected with TRAIL siRNA as compared with cells transfected with control siRNA. Importantly, we found that cells transfected with TRAIL siRNA were more resistant to MS275, Adriamycin, or their combination, as compared with cells transfected with control siRNA (Fig. 5*C*), suggesting that TRAIL is important for cell death induced by such treatments. Because TRAIL is a potent apoptosis inducer, we investigated the effects of down-regulation of TRAIL on MS275-mediated Adriamycin-induced apoptosis. Cells transfected with TRAIL or control siRNA were treated with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 48 hours, and the activation of the apoptotic pathways was then examined. As shown in Fig. 5*B*, cleavage of caspase-9, caspase-8, caspase-3, and PARP was significant in cells treated with both MS275 and Adriamycin, as compared with untreated or cells treated with MS275 alone, whereas such changes were minimal in Adriamycin-treated cells. In contrast, cleavage of caspase-9, caspase-8, caspase-3, and PARP was decreased in cells transfected with TRAIL siRNA following the combined treatment (Fig. 5*B*). Additionally, we found that such treatments increased caspase-3 activity, which was abolished in cells transfected with TRAIL siRNA (data not shown), indicating that down-regulation of TRAIL impairs the activation of the caspase cascade induced by the combined treatment, thereby enhancing cell survival. Collectively, these results suggest that TRAIL plays an important role in MS275-mediated Adriamycin-induced apoptosis.

Role of Sp1 in chemosensitivity. We have shown that TRAIL expression is regulated by Sp1. Because TRAIL induction plays an important role in apoptosis (26, 27), we asked whether Sp1 could substitute TRAIL to induce apoptosis by MS275 in the presence or absence of Adriamycin. To answer this question, we transfected T47D cells with Sp1 or control siRNA and then treated transfected cells with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 48 hours, and the growth inhibition was then examined. As shown in Fig. 6*A*, the levels of Sp1 in cells transfected with Sp1 siRNA were significantly decreased as compared with cells transfected with control siRNA. Interestingly, we did not detect the changes in the levels of Sp1 protein in response to the treatments (Fig. 6*A*), suggesting that increased Sp1 transcription activity (Fig. 4) may not be due to an increase in the total level of Sp1 protein. However, we found that induction of TRAIL by MS275 was abolished in Sp1 siRNA–transfected cells as compared with control siRNA–transfected cells. Furthermore, the induction of TRAIL by the combination was also decreased when Sp1 was down-regulated (Fig. 6*A*). These data indicate that induction of TRAIL by MS275 alone or in combination with Adriamycin is dependent on the presence of Sp1.

To determine the effect of Sp1 knockdown on cell viability, T47D cells transfected with either Sp1 or control siRNA were treated with MS275, Adriamycin, or their combination for 48 hours, and cell viability was then determined. Figure 6*C* shows that cells transfected with Sp1 siRNA are more resistant to MS275, Adriamycin, or their combination, as compared with cells transfected with control siRNA, which is similar to the results obtained with cells in which TRAIL was down-regulated (Fig. 5*C*). These data suggest that Sp1-dependent TRAIL expression is critical for cell death induced by MS275, Adriamycin, or their combination.

To investigate the effects of down-regulation of Sp1 on MS275-mediated Adriamycin-induced apoptosis, cells transfected with Sp1 or control siRNA were treated with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 48 hours, and activation of caspases and cleavage of PARP was examined. As expected, in cells transfected with control siRNA, cleavage of caspase-9, caspase-8, caspase-3, and PARP was significantly increased in cells that received the combined treatments, as compared with untreated cells, whereas such changes were minimal in either agent-treated

cells (Fig. 6*B*). In contrast, such changes were decreased in cells transfected with Sp1 siRNA following the combined treatment (Fig. 6*B*), indicating that down-regulation of Sp1 decreases activation of the caspase cascade induced by the combined treatment, leading to improved cell survival. Taken together, these results suggest that Sp1 plays an important role in MS275-mediated Adriamycin-induced caspase activation and apoptosis.

Role of Sp1 in chemosensitivity in Sp1-knockout mouse embryonic stem cells. We have shown that down-regulation of Sp1 by siRNA decreases T47D cell death induced by the combined treatment with MS275 and Adriamycin (Fig. 6*C*). Because Sp1 expression could not be completely eliminated by siRNA silencing (Fig. 6*A*), the results obtained with an siRNA approach may not completely reflect the role of Sp1 in chemosensitivity. To overcome this difficulty, we examined the role of Sp1 in chemosensitivity using Sp1-knockout mouse embryonic stem cells. As expected, full-length Sp1 was expressed in $Sp1^{+/+}$ embryonic stem cells but not in $Sp1^{-/-}$ embryonic stem cells (Fig. 6*D, top*), confirming Sp1 deletion in $Sp1^{-/-}$ cells. We then treated these cells with MS275, Adriamycin, or their combination, and the effects of such treatments on cell death were assessed. As shown in Fig. 6*D* (*bottom*), $Sp1^{-/-}$ cells were more resistant than $Sp1^{+/+}$ cells to MS275 or Adriamycin; there were ~57% and ~74% of surviving $Sp1^{-/-}$ cells as compared with ~43% and ~42% of surviving $Sp1^{+/+}$ cells following the treatments with MS275 and Adriamycin, respectively. More importantly, we showed that the combined treatment results in a significant increase in cell survival of $Sp1^{-/-}$ cells as compared with $Sp1^{+/+}$ cells (42% versus 18%). In addition, we showed that TRAIL is induced in $Sp1^{+/+}$ but not in $Sp1^{-/-}$ cells by the combined treatments (Fig. 6*D, top*), further confirming the requirement of Sp1 for TRAIL induction by the combined treatment. Thus, these results suggest that Sp1 plays a critical role in cell death induced by combined treatment with MS275 and Adriamycin, which may be through the induction of TRAIL and activation of the TRAIL apoptotic pathway.

Discussion

In this study, we show that MS275 induces TRAIL, which sensitizes human breast cancer T47D cells and mouse embryonic stem cells to Adriamycin-induced death. We also show that the underlying mechanism of such sensitization is mediated by the transcription factor Sp1 because Sp1 knockdown or deletion abolishes TRAIL induction and subsequently renders cells resistant to MS275, Adriamycin, or their combination. Thus, our findings indicate for the first time that the transcription factor Sp1 plays a critical role in chemosensitivity and thereby is a potential therapeutic target for the development of novel anticancer agents.

Sp1 was the first mammalian transcription factor to be identified (35). It binds to GC-rich sequences to regulate gene expression (36). Although Sp1 is a basal transcription factor, recent studies suggested that it plays an important role in tumor growth and metastasis. For example, Sp1 is overexpressed in both gastric and pancreatic cancers, and overexpression of Sp1 enhances the expression of vascular endothelial growth factor, which promotes cancer angiogenesis (37, 38). However, it is not known whether Sp1 plays a role in the responses of cancer cells to chemotherapy. In this study, we showed that knockdown of Sp1 in T47D cells abolishes cell death induced by MS275, Adriamycin, or their combination. We also showed that knockdown of Sp1 in T47D cells substantially decreases the activation of the caspase cascade induced by the combined treatments, suggesting that Sp1 knockdown impairs caspase-mediated cell death induced by chemotherapeutic agents. Furthermore, we showed that Sp1 knockout decreased mouse embryonic stem cell death induced by MS275, Adriamycin, or their combination, particularly for the combined treatments. However, it is not clear whether Sp1 plays a role in sensitizing other cancer cells to additional anticancer agents, which requires further investigation. Additionally, although Sp1 is a basal transcription factor and has many target genes, it is possible that in some cells after chemotherapeutic drug treatment, Sp1 might preferentially activate some apoptosis-related genes (e.g.,

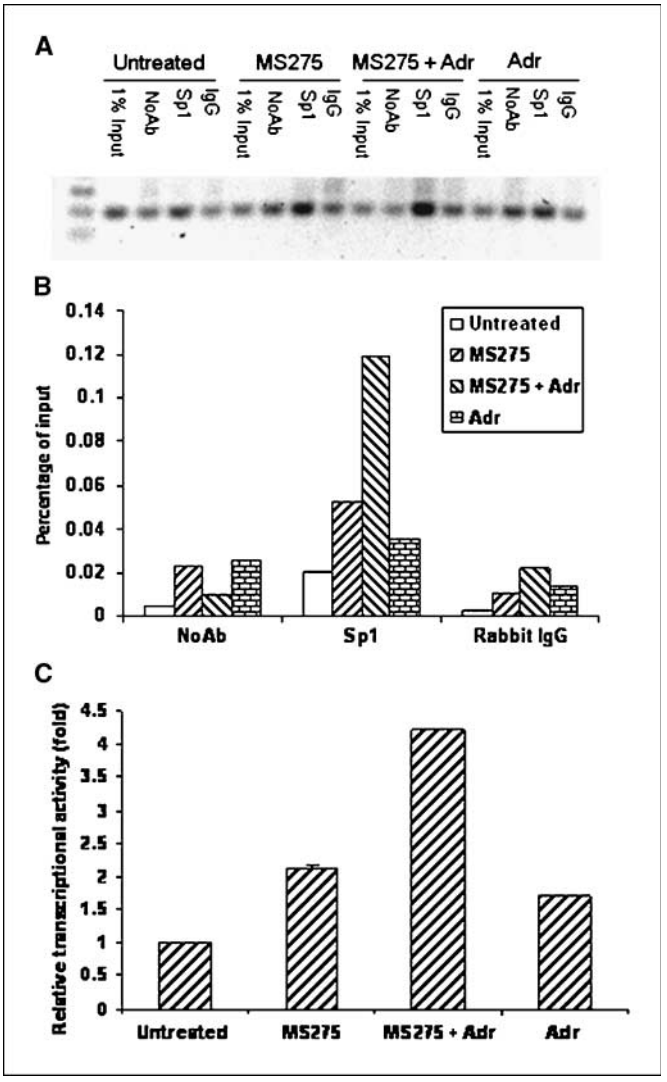

Figure 4. Role of Sp1 in TRAIL induction by MS275, Adriamycin, or their combination. *A* and *B, in vivo* binding of Sp1 to the TRAIL promoter by chromatin immunoprecipitation assays. T47D cells were treated with MS275, Adriamycin, or their combination for 24 h. Chromatin immunoprecipitation was done using the ChIP Assay Kit as described in Materials and Methods. Immunoprecipitations were done with rabbit polyclonal antibodies against Sp1. Immunoprecipitations with rabbit IgG were used as negative controls. *NoAb,* no antibody. The amplified DNA fragments for Sp1 sites are shown in *A*. Quantification of amplified DNA is shown in *B*. The results are presented as percentages of DNA immunoprecipitated relative to the input. *C,* ELISA assay for Sp1 transcription activity. T47D cells were left untreated or treated with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 24 h and nuclear protein was extracted with a Nuclear Extraction kit. A total of 15 μg of nuclear protein from each treatment was analyzed for Sp1 activity using the Transcription Factor ELISA kit. Absorbance was obtained with a spectrophotometer at 450 nm. Relative transcriptional activity in untreated cells was arbitrarily given as 1.

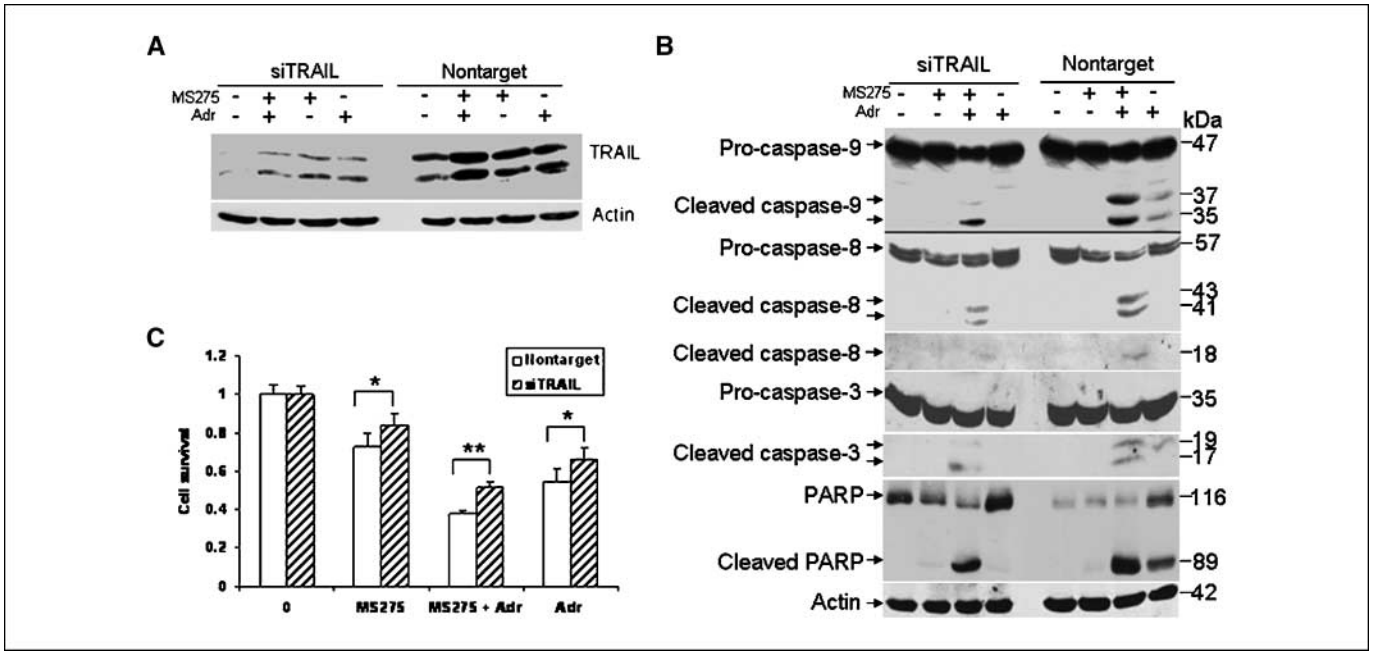

Figure 5. Effect of TRAIL down-regulation by siRNA on cell death induced by MS275, Adriamycin, or their combination. *A,* down-regulation of TRAIL by siRNA silencing. T47D cells were plated at 6×10^5 per well in six-well plates. Cells were transfected with TRAIL (*siTRAIL*) or nontarget siRNA. After 3 d, cells were left untreated or treated with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 48 h, and induction of TRAIL was determined by Western blot analysis. *B,* effect of down-regulation of TRAIL by siRNA on the caspase cascade. T47D cells were transfected with TRAIL or nontarget siRNA and treated with MS275 (5 μmol/L) with and without Adriamycin (0.1 μg/mL) for 48 h as described in *A*. Total protein was extracted for assaying cleavage of caspase-9, caspase-8, caspase-3, and PARP by Western blot analysis. β-Actin was included as a loading control. *C,* effect of silencing TRAIL expression on cell survival. T47D cells were transfected with TRAIL or nontarget siRNA as described in *B*. After 3 d, cells were left untreated or treated with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 48 h. Cell viability was determined by MTT assays. Cell survival data are expressed as percentage of untreated cells. Representative of three independent experiments. **, $P < 0.01$; *, $P < 0.05$.

TRAIL) to induce cell death. Because Sp1 is overexpressed in some cancers (37, 39), anticancer drugs that can activate Sp1-dependent TRAIL expression might be effective against these tumors, and this requires further investigation. Nevertheless, this study provides the first proof-of-principal to target Sp1 for cancer therapy.

What are the underlying mechanisms by which Sp1 favors cell death induced by test agents? We believe that Sp1 could transcriptionally activate TRAIL, leading to the activation of the TRAIL apoptotic pathway because knockdown of Sp1 decreased TRAIL induction and subsequently increased chemoresistance. Although we showed that knockdown of Sp1 could decrease the chemosensitivity of breast cancer T47D cells to MS275, Adriamycin, or their combination (Fig. 6*C*), the effect of Sp1 knockdown on decreased chemosensitivity in T47D was not as evident as observed in Sp1-knockout embryonic stem cells (Fig. 6*D*). This difference may be due to the level of Sp1 protein. Because Sp1 is highly expressed in T47D cells, siRNA silencing can substantially down-regulate, but not completely eliminate, Sp1 expression (Fig. 6*A*). The remaining Sp1 may contribute to the observed cell death in Sp1 knockdown T47D cells. Consistent with this, we showed that more surviving cells were observed in $Sp1^{-/-}$ as compared with $Sp1^{+/+}$ embryonic stem cells presumably because Sp1 was completely eliminated in $Sp1^{-/-}$ cells (Fig. 6*D*).

It has been shown that protein modifications including phosphorylation by anticancer agents increase Sp1 transcription activity (40). Furthermore, Sp1 can be cleaved, and cleaved Sp1 has a higher activity (41). Because treatment of either agent alone or in combination had no effects on the levels of Sp1 protein, we believe that TRAIL expression induced by the treatments may not be due to the alteration of the level of Sp1 protein. Our preliminary data indicated that Adriamycin, MS275, or their combination could cause Sp1 cleavage (data not shown), suggesting that Sp1 cleavage by the treatments may enhance Sp1 activity, leading to increased TRAIL expression, which is under investigation. In addition, it has been shown that HDAC1 could interact with Sp1 to regulate its activity (42). Therefore, we tested the effect of MS275 on Sp1 and HDAC1 interaction by immunoprecipitation/Western blot. Although HDAC1 activity was inhibited, MS275 had no effect on the interaction of Sp1 with HDAC1 (data not shown). Because there are several HDAC family members, it is possible that MS275 could affect other HDAC members to regulate Sp1 activity. Regardless, we showed that MS275 alone or in combination with Adriamycin increased Sp1 transcription activity.

Currently, there are few agents that are truly cancer cell specific in terms of efficacy and induction of cell death. TRAIL is an example of a molecule that selectively kills transformed and cancer cells but not most normal cells (14). Although TRAIL can selectively kill tumor or transformed cells without harming normal cells, its regulation in tumors, particularly in solid tumors, is not fully understood. Previous studies identified several regulatory elements in TRAIL promoter, which include IFN-stimulated response element, nuclear factor κB, and Sp1 (18–20). We previously showed that TRAIL is induced by TNFα and 5-aza-2′-deoxycytidine through distinct mechanisms (33, 34). In this study, we showed that deletion of the second Sp1 binding site results in a complete loss of TRAIL promoter activity induced by

MS275 alone or in combination with Adriamycin, indicating that this Sp1 site is required for transactivation of the TRAIL promoter by our test agents. Furthermore, we have shown by chromatin immunoprecipitation assays that Sp1 can bind to the TRAIL promoter on the treatments. Taken together, we conclude that the second Sp1 site is important for the activation of the TRAIL promoter.

We have shown that Adriamycin can induce TRAIL at the protein level but had no effect on the TRAIL promoter activity. We have also shown that Adriamycin can enhance the TRAIL promoter activity induced by MS275. This suggests that Adriamycin induces other factors that may indirectly affect the activity of the TRAIL promoter. Consistent with this, we have shown by ELISA that there is a slight increase in TRAIL promoter activity induced by Adriamycin treatment alone (Fig. 4*C*).

Defective apoptotic responses are hallmarks of cancer cells, and apoptotic pathways are therefore attractive therapeutic targets. In addition to the use of TRAIL and agonistic antibodies alone or in combination with clinical chemotherapeutic agents, several new compounds, including HDAC inhibitors that target the apoptotic pathway, are under development (26, 27). Although HDAC inhibitors can activate transcription of target genes via histone acetylation to kill cancer cells by cell cycle arrest and apoptosis (24), recent studies indicated that induction of TRAIL plays a critical role in leukemia cell death (26, 27). Similarly, we showed here that HDAC inhibitors induce TRAIL in breast cancer cells (Fig. 1) and that such induction is critical for T47D and mouse embryonic stem cell death induced by Adriamycin (Fig. 6*C* and *D*). Thus, our study suggests that induction of endogenous TRAIL sensitizes cancer cells to chemotherapy.

In summary, we show that HDAC inhibitors induce TRAIL via the second Sp1 binding site in the promoter of the *TRAIL* gene. We also show that the treatment with MS275 sensitizes breast cancer cells or mouse embryonic stem cells to Adriamycin-induced death. More importantly, we show that Sp1-dependent TRAIL induction plays a critical role in cell death induced by combined treatments with MS275 and Adriamycin because down-regulation of TRAIL by siRNA in T47D or deletion of Sp1 in Sp1-knockout mouse

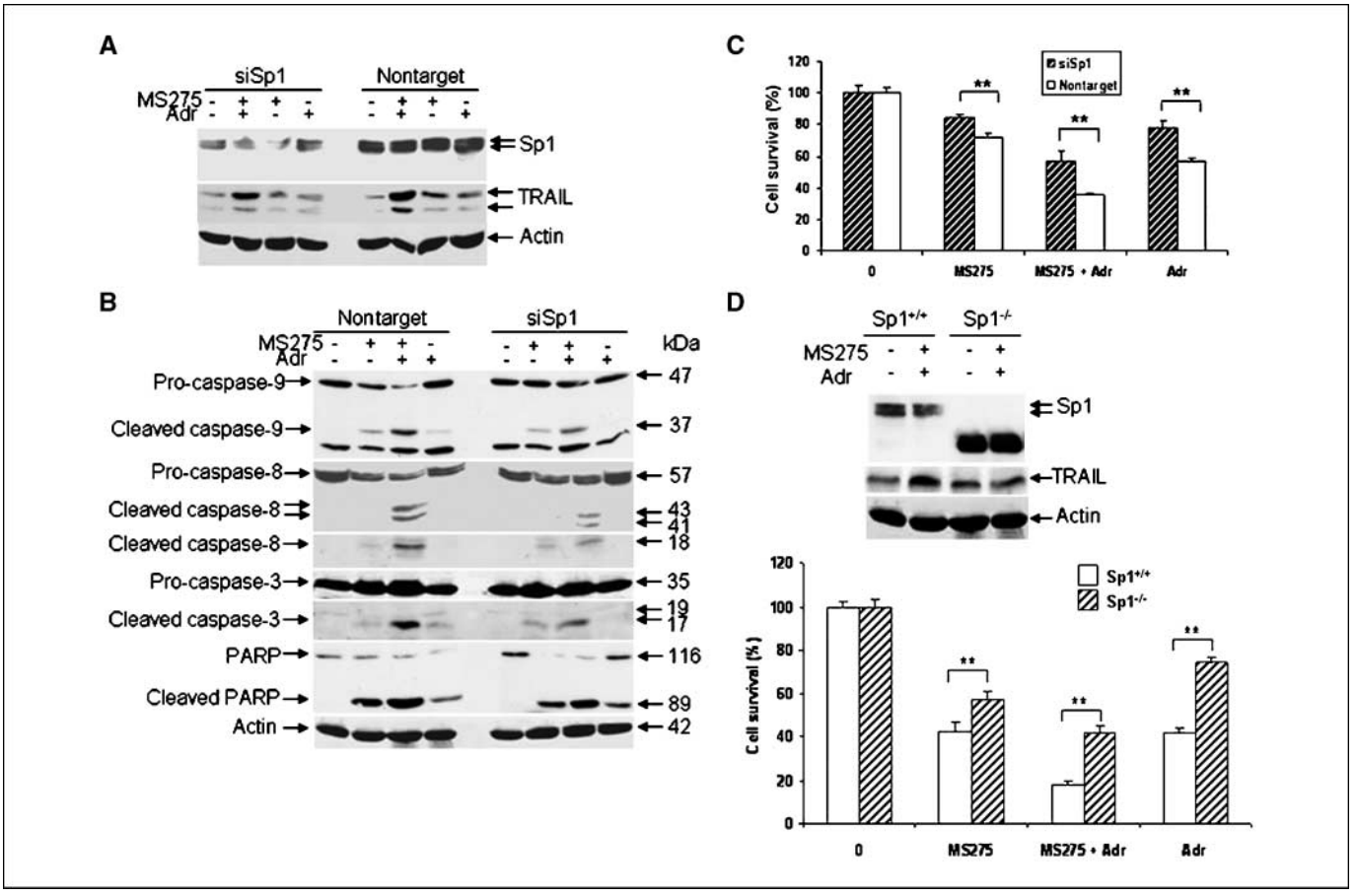

Figure 6. Role of Sp1 in cell death induced by MS275, Adriamycin, or their combination. *A,* knockdown of Sp1 by siRNA silencing. T47D cells were plated at 6×10^5 per well in six-well plates. The next day, cells were transfected with Sp1 or nontarget siRNA. Cells were left untreated or treated with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 48 h, and induction of TRAIL and Sp1 was determined by Western blot analysis. *B,* effect of down-regulation of Sp1 (*siSp1*) by siRNA on the caspase cascade. T47D cells were transfected with Sp1 or nontarget siRNA and treated with MS275 (5 μmol/L) in the presence or absence of Adriamycin (0.1 μg/mL) for 48 h. Total protein was extracted for assaying cleavage of caspase-9, caspase-8, caspase-3, and PARP by Western blot analysis. β-Actin was included as a loading control. *C,* effect of silencing Sp1 expression on cell survival. T47D cells were transfected with Sp1 or nontarget siRNA. Cells were left untreated or treated with MS275 (5 μmol/L), Adriamycin (0.1 μg/mL), or their combination for 48 h and cell viability was determined by MTT assays. Cell survival data are expressed as percentage of untreated cells. Representative of three independent experiments. **, $P < 0.01$; *, $P < 0.05$. *D,* effect of Sp1 depletion on chemosensitivity. *Top,* $Sp1^{+/+}$ and $Sp1^{-/-}$ embryonic stem cells were treated with both MS275 (5 μmol/L) and Adriamycin (0.1 μg/mL) for 24 h. Total protein was extracted and then assayed for the levels of TRAIL and Sp1 by Western blot analysis. β-Actin was used as a loading control. *Bottom,* $Sp1^{+/+}$ and $Sp1^{-/-}$ embryonic stem cells were treated with MS275 (3 μmol/L), Adriamycin (0.01 μg/mL), or their combination for 48 h, and cell viability was determined by MTT assays. Cell viability data are expressed as percent of untreated cells. Representative of three independent experiments. **, $P < 0.01$; *, $P < 0.05$.

embryonic stem cells abolishes such sensitization. Therefore, our findings suggest that Sp1 is a new target in human cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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